

Purification and Characterization of Thermostable Direct Hemolysin of *Vibrio parahaemolyticus*

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A thermostable direct hemolysin was purified from culture filtrates of *Vibrio parahaemolyticus*. The purified hemolysin gave one precipitation line with the antihemolysin antiserum on agar-gel diffusion test and a single band on polyacrylamide gel electrophoresis. The hemolysin was not inactivated by heating at 70 to 100 C for 10 min. The hemolytic activity was not enhanced by the addition of lecithin. It was demonstrated that the hemolysin was a protein with a molecular weight of approximately 118,000. Amino acid analysis revealed that 43% of total amino acids were acidic amino acids, whereas 11% were basic amino acids.

Vibrio parahaemolyticus produces thermolabile and thermostable direct hemolysins (7, 14, 18). In addition, existence of indirect hemolysin has been reported (17). Among these hemolysins, thermostable direct hemolysin was reported to be closely correlated with human pathogenicity (14). To study the role of thermostable direct hemolysin in human infection of *V. parahaemolyticus*, it is necessary to get a highly purified thermostable direct hemolysin. Although attempts have been made to purify the hemolysin of *V. parahaemolyticus* (12, 18), high purification of the hemolysin has been so far unsuccessful. In this paper, we report the extensive purification and some characters of thermostable direct hemolysin of *V. parahaemolyticus*.

MATERIALS AND METHODS

Preparation of partially purified thermostable direct hemolysin. Crude hemolysin of *V. parahaemolyticus* WP-1 was prepared as described previously (12). It was then purified by diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex A-50 column chromatography as described in a previous paper (12). Eluates from the last DEAE-Sephadex A-50 column were designated as fraction A-50.

Determination of hemolytic activity. Hemolytic activity of the hemolysin was determined as described previously (12). A 0.25-ml sample of the fraction was serially diluted with 0.01 M phosphate buffer (pH 7.0) containing 0.9% sodium chloride. An equal volume of 1% suspension of human washed erythrocytes in 0.9% sodium chloride solution (about 8×10^7 cells/ml) was

added. After the mixtures were incubated at 37 C for 2 h, they were kept at 4 C for 12 h. Hemolytic activity was estimated by the degree of hemolysis observed.

Agar-gel diffusion test. The plate method of Ouchterlony (16) was employed as described previously (11).

Preparation of antihemolysin antiserum. Antihemolysin antiserum was prepared by immunizing rabbits with the crude hemolysin. Freund incomplete adjuvant was used for preparation of immunizing antigens, and two intramuscular injections of the crude hemolysin (containing about 20 mg of protein) were carried out at 2-week intervals. After 2 weeks, an intravenous booster injection of the crude hemolysin solution was made. Antiserum was obtained 1 week after the last injection.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the purified hemolysin was carried out as described by Davis (6). About 10 μ g of the purified hemolysin was mixed with the sample gel and placed on top of the gel. Electrophoresis was carried out at 4 C for 4 h at a constant current of 2 mA per tube. One gel was stained by Amido Black 10 B. The other gel was cut into strips 2 mm wide, and the hemolysin was extracted from each piece with 1 ml of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0) to assay the hemolytic activity.

Determination of carbohydrate content. The carbohydrate content was determined with Dreywood anthrone reagent as described by Morris (15).

Determination of molecular weight by gel filtration. Molecular weight was estimated by gel filtration by the method of Andrews (2) by use of a Sephadex G-200 column (2.5 cm by 35 cm). Equilibration and elution of the column were done with 0.01 M

phosphate buffer (pH 7.0). Catalase (EC 1.11.1.1), alpha amylase (EC 3.2.1.1), beta galactosidase (EC 3.2.1.23), and human gamma globulin were used as standards. The standard proteins were assayed by their absorbance at 280 nm, and the hemolysin was assayed by its hemolytic activity.

Determination of molecular weight by sedimentation velocity. The sedimentation velocity method in sucrose density gradient was carried out as described by Martin and Ames (10). The hemolysin was centrifuged in a linear sucrose density gradient (5 to 25%) in an SW50.1 rotor at 32,000 rpm for 17 h. Human gamma globulin and alpha amylase were used as standards.

Amino acid analysis. The amino acid composition of the purified hemolysin was determined by use of a Nihon-Denshi (model JLC-5AH) amino acid analyzer. A sample (500 μ g) was hydrolyzed by heating in 2 ml of 6 N HCl at 105 C for 16 h in a sealed tube. Then, the mixture was dried by evaporation and dissolved in 2 ml of 0.2 M citrate buffer (pH 2.2). A 0.8-ml sample was examined in the analyzer.

RESULTS

Purification of thermostable direct hemolysin. The purification of partially purified thermostable direct hemolysin was carried out as follows. About 5 mg of fraction A-50 was incubated in 19 ml of 0.01 M phosphate buffer (pH 7.0) containing 4 M urea and 2 M 2-mercaptoethanol for 30 min at 50 C. The reaction mixture was then dialyzed against 0.01 M phosphate buffer (pH 7.0) containing 12 mM 2-mercaptoethanol at 4 C for 48 h. After concentration, the urea-treated fraction was applied to a Sephadex G-200 column (2 cm by 35 cm) and eluted with phosphate buffer (0.01 M, pH 7.0) containing 12 mM 2-mercaptoethanol (Fig. 1). The urea-treated fraction was separated into three peaks by Sephadex G-200 column chromatography. The hemolytic activity was recovered with fraction P-II, whereas both fraction P-I and P-III contained no hemolytic activity. A typical purification procedure is summarized in Table 1.

The results of the agar-gel diffusion test are presented in Fig. 2. Fraction A-50 gave two precipitation lines with antihemolysin antiserum prepared with the crude hemolysin. Fraction P-II shared one of these two precipitation lines.

Purity of the purified thermostable direct hemolysin was tested by polyacrylamide gel electrophoresis. As shown in Fig. 3A, the purified thermostable direct hemolysin gave a single band on polyacrylamide gel electrophoresis. Coincidence of hemolytic activity with this band was demonstrated by eluting out the hemolysin of the gel and assaying the hemolysin eluted (Fig. 3B).

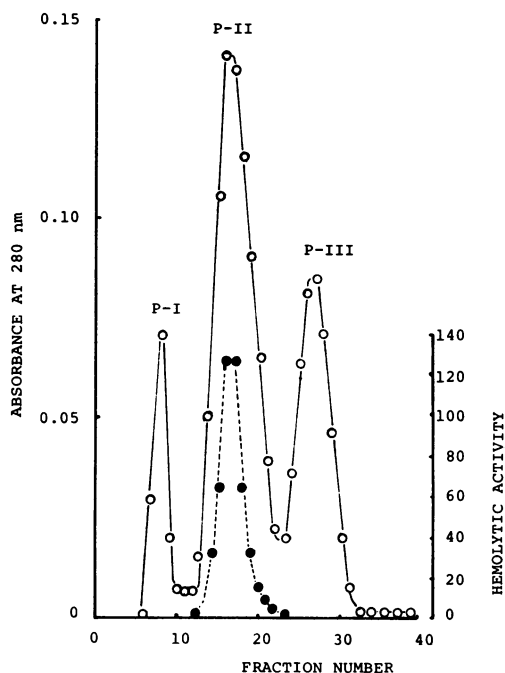


FIG. 1. Sephadex G-200 column chromatography of urea-treated fraction A-50. Partially purified thermostable direct hemolysin of *V. parahaemolyticus* (fraction A-50) was treated by urea as described in the text. Chromatography on Sephadex G-200 of urea-treated fraction A-50 was carried out as described in the text, and each fraction was collected in 5-ml samples. Hemolytic activity was determined as described in the text and expressed as the reciprocal of the highest dilution which gave complete hemolysis. Symbols: O, absorbance at 280 nm; ●, hemolytic activity.

Characters of the purified hemolysin. The heat stability of the purified hemolysin was studied (Table 2). The purified hemolysin was not inactivated by heating at 70 to 100 C for 10 min, indicating the hemolysin was thermostable.

Experimental data of the effects of lecithin on the hemolytic activity of the purified hemolysin are presented in Table 3. The hemolytic activity was not enhanced by the addition of lecithin, indicating that the hemolysin was not indirect, but was direct, hemolysin.

Physicochemical properties of the purified thermostable direct hemolysin. The purified thermostable direct hemolysin was treated with various proteinases such as pepsin (EC 3.4.4.1), trypsin (EC 3.4.4.4), alpha chymotrypsin, nargase, and papain (EC 3.4.4.10). The hemolytic activity of the purified thermostable direct hemolysin was almost completely destroyed by pepsin, trypsin, alpha chymotrypsin, and na-

TABLE 1. Purification of thermostable direct hemolysin of *V. parahaemolyticus* WP-1

Fraction	Total volume (ml)	Total protein ^a (mg)	Total activity ^b (HU)	Specific activity (HU/mg)	Relative activity	Yield (%)
Ammonium sulfate fraction	670	15,812	5,360	0.339	(1.0)	100
Acid precipitate	82	1,927	4,920	2.55	7.5	92
1st DEAE-cellulose column eluate	30	159	3,960	24.9	73.4	74
2nd DEAE-cellulose column eluate	20	56	2,000	35.7	105	37
DEAE-Sephadex A-50 column eluate	2.3	8.1	662	82.3	243	12
Sephadex G-200 column eluate	1.0	3.4	528	155	457	9.9

^a Protein content is measured by the method of Lowry et al. (9).

^b A 0.5-ml sample of hemolysin fraction and 1.5 ml of a 1% suspension of erythrocytes in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl were incubated at 37 C for 1 h. After the reaction mixtures were centrifuged at 3,000 rpm for 15 min, the hemolytic activity was determined by measuring the absorbance at 540 nm of the resulting supernatant fluid. One unit of hemolysin causes 50% hemolysis of the suspension for 1 h at 37 C. HU, Hemolytic unit.

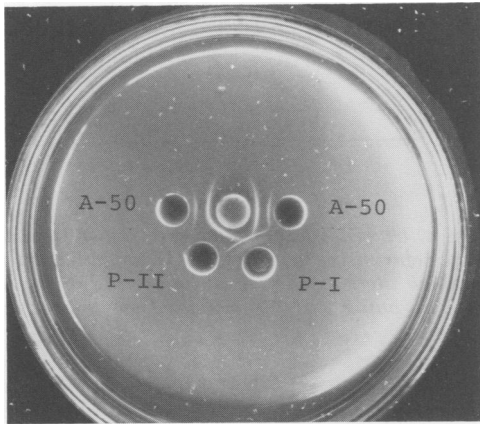


FIG. 2. Agar-gel diffusion test of fraction A-50, P-I, and P-II. The agar-gel diffusion test was carried out as described in the text. Anti-hemolysin antiserum was placed in the center well.

garse, suggesting that the hemolysin has a protein nature (Table 4).

The ultraviolet absorption spectrum of the purified thermostable direct hemolysin (Fig. 4) also gave a typical profile of protein, a maximum and a minimum absorption being 277 nm and 250 nm, respectively. No carbohydrate was detected in about 400 μ g of the purified hemolysin. It was found that the purified hemolysin was completely precipitated at pH values below 4.0 and no hemolytic activity remained in the supernatant fluid after centrifugation at 30,000 rpm for 60 min. When pH of the solution was elevated to 5.0, most hemolytic activity was not precipitated and remained in the supernatant fluid. This suggests that the isoelectric point of the hemolysin was between pH 4 and 5.

The molecular weight of the purified hemolysin was determined as described in the text. The

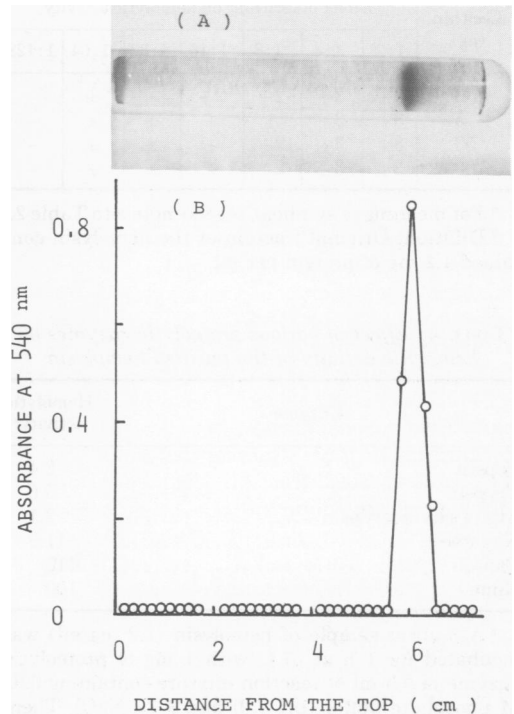


FIG. 3. Polyacrylamide gel electrophoresis of purified thermostable direct hemolysin. Polyacrylamide gel electrophoresis was carried out as described in the text. A, The gel was stained by Amido Black 10 B. Decolorization was done with 7% acetic acid. B, the gel was cut into strips 2 mm wide and suspended in 1.5 ml of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0) containing 0.9% NaCl. An equal volume of a 1% suspension of human erythrocytes was added and incubated at 37 C for 2 h. After centrifugation, hemolytic activity was determined spectrophotometrically as described in footnote of Table 4.

TABLE 2. *Thermostability of the purified hemolysin*

Heat treatment	Thermostability ^a							
	1:2 ^b	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Untreated	3+	3+	3+	2+	+	+	±	—
70 C, 10 min	3+	3+	3+	2+	+	+	±	—
100 C, 10 min	3+	3+	3+	2+	+	+	—	—

^a Symbols: 3+, complete hemolysis; 2+, +, ±, degree of partial hemolysis; —, no hemolysis.

^b Dilution. Original fraction of the hemolysin contained 1.2 mg of protein per ml.

TABLE 3. *Lack of effect of lecithin on hemolytic activity of the purified hemolysin*

Lecithin (μg)	Effect of lecithin ^a on hemolytic activity						
	1:2 ^b	1:4	1:8	1:16	1:32	1:64	1:128
0	3+	3+	2+	2+	+	±	—
5	3+	3+	+	+	±	±	—
50	3+	3+	2+	+	±	±	—
100	3+	3+	2+	+	±	±	—

^a For meaning of symbols, see footnote *a* to Table 2.

^b Dilution. Original fraction of the hemolysin contained 1.2 mg of protein per ml.

TABLE 4. *Effect of various proteolytic enzymes on hemolytic activity of the purified hemolysin*

Enzyme	Hemolytic activity ^a
Pepsin	2.4
Trypsin	3.6
Alpha chymotrypsin	2.1
Nagarse	11.9
Papain	110
None	100

^a A 5-μliter sample of hemolysin (1.2 mg/ml) was incubated for 1 h at 37 C with 1 mg of proteolytic enzyme in 0.5 ml of reaction mixture containing 0.01 M phosphate buffer (pH 7.0) and 0.9% NaCl. Then, 2.5 ml of a 1% suspension of erythrocytes in 0.9% NaCl solution was added and incubated at 37 C for 2 h. After the reaction mixtures were centrifuged at 3,000 rpm for 15 min, hemolytic activity was determined by measuring the absorbance at 540 nm of the resulting supernatant fluid. Figures were expressed as percentages of hemolytic activity of the hemolysin in the absence of proteolytic enzymes.

molecular weight of the hemolysin was estimated as approximately 118,000 by the gel filtration method (Fig. 5). To substantiate the result obtained from gel filtration, an alterna-

tive method using a sucrose density gradient was employed and it too gave a value of approximately 114,000.

Experimental results of amino acid analysis

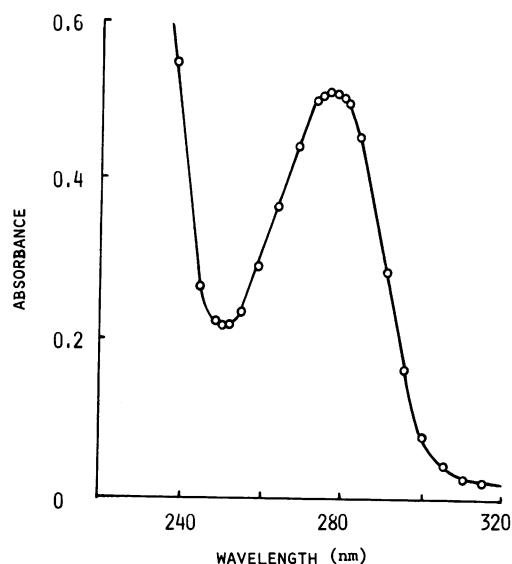


FIG. 4. Ultraviolet absorption spectrum of purified thermostable direct hemolysin. A 1.2 mg/ml solution of purified thermostable direct hemolysin in 0.01 M phosphate buffer (pH 7.0) was used to measure the absorbance.

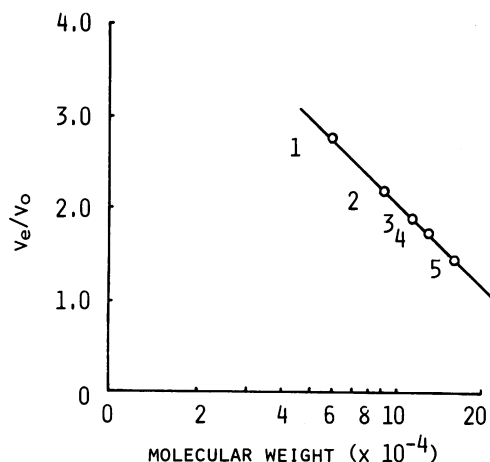


FIG. 5. Determination of molecular weight by Sephadex G-200 gel filtration. Determination of molecular weight of purified thermostable direct hemolysin by Sephadex G-200 gel filtration was carried out as described in the text. V_o is void volume of the column determined with Blue Dextran 2000 and V_e is elution volume of each substance. 1, Catalase; 2, alpha amylase; 3, purified thermostable direct hemolysin; 4, beta galactosidase; 5, human gamma globulin.

of the purified hemolysin are presented in Table 5. Approximately 43% of the total amino acids were acidic amino acids, whereas only 11% were basic amino acids.

DISCUSSION

Since close correlation of thermostable direct hemolysin of *V. parahaemolyticus* with human pathogenicity was reported by Miyamoto et al. (14), attempts to purify the hemolysin have been reported. Zen-Yoji et al. (18) reported a partial purification and characterization of thermostable hemolysin. We also reported previously a partial purification procedure of thermostable direct hemolysin (12). However, these hemolysins were not sufficiently pure to study the physicochemical and biological nature of the hemolysin. By using Sephadex G-200 column chromatography after urea treatment of partially purified thermostable direct hemolysin, we succeeded in purifying the hemolysin to a high degree.

The purified hemolysin gave a single band on polyacrylamide gel electrophoresis. It had the nature of a protein, and no carbohydrates were detected.

The molecular weight of the purified thermostable direct hemolysin was determined to be approximately 118,000. This value is close to the molecular weight of staphylococcal delta toxin (8), but larger than that of various he-

molysins produced by other bacteria, such as staphylococcal alpha toxin (4), beta toxin (5), and streptococcal hemolysin (1, 3).

The hemolysin treated by either urea or sodium lauryl sulfate at 50 C for 30 min gave the same behavior as the untreated hemolysin on polyacrylamide gel electrophoresis. When culture filtrates containing hemolysins were analyzed by sucrose density gradient centrifugation and by Sephadex gel filtration, profiles of hemolytic activity were the same as that given by the purified hemolysin (unpublished observation). These results suggest that the purified thermostable direct hemolysin did not consist of multiple subunits.

The purified thermostable direct hemolysin of *V. parahaemolyticus* contained a large amount of acidic amino acid, approximately 43% of the total amino acids. This was consistent with the experimental results that the isoelectric point of the hemolysin was between pH 4.0 and 5.0.

In crude hemolysins prepared from *V. parahaemolyticus*, the existence of an Arrhenius effect was reported (13). This effect was not demonstrated with the purified hemolysin, suggesting that causative factor(s) of the Arrhenius effect was released from the hemolysin during the purification.

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TABLE 5. Amino acid analysis of the purified hemolysin

Amino acid	Amt of amino acid found ($\mu\text{mol} \times 10^{-3}$) ^a	Amino acid residues (%) ^b
Asp	2.92	14.9
Thr	1.35	6.9
Ser	2.43	12.4
Glu	1.76	9.0
Pro	0	0
Gly	1.35	6.9
Ala	1.02	5.2
Cys	Trace	
Val	2.21	11.3
Met	0.36	1.8
Ileu	0.63	3.2
Leu	0.76	4.0
Tyr	1.15	5.9
Phe	1.33	6.9
Lys	1.39	7.1
His	0.54	2.8
Arg	0.34	1.7
CysSO ₃ H	Trace	

^a Recovery of amino acids from 247 μg of protein hydrolyzed.

^b Total of individual amino acid residues is taken as 100.0%.

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